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The application of new breeding strategy for tolerance to drought, resistance to Hessian fly, resistance to rust and end-use quality of protein content in bread wheat (*Triticum aestivum* L.)

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Abstract

Genetic diversity in crop specie is essential to breed buffered genotypes capable to withstand under biotic and abiotic stress conditions. An approach called *genotypic selection* based on the widespread conventional selection with the use of information of the molecular markers can facilitate breeding strategy by providing effective achievement of biotic stress resistance reducing in mean time generation interval and investments in ecological-friendly crop production is reviewed. Also the *phenotypic selection* is an important step in breeding programs, and genetic variability increases the chances of obtaining variance in progenies. In this study, we present a practical validation of the breeding strategy to produce bread wheat lines derived from a three elite cultivar with superior dough properties and durable rust resistance. Molecular markers were used to screen a double hybrid population produced from a cross between the three varieties of bread wheat considered as donor parents: *Dharwar*, *Annuello* and *Stylet* crossed with six varieties considered as recurrent parents: *Achtar*, *Aguilal*, *Merchouch*, *Baraka*, *Salama* and *Amal*. Following the phenotypic selection was applied for the doubled haploid plants to select new genotypes for rust resistance, Hessian fly resistance, drought tolerance and grain protein content.

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Introduction

In Morocco, bread wheat (*Triticum aestivum* L.) occupies, in both production and area, an important position, but the productivity is affected by various biotic and abiotic stresses. Developing new wheat varieties using the breeding program is the most effective means to managing these stresses and improving the productivity (El Haddoury *et al.,* 2012). The objectives of the breeding strategy used in this experiment is to develop new bread wheat variety with different quality, as rust resistance, Hessian fly (HF) resistance, drought tolerance and end-use quality of a gluten protein.

The HF, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), has been recognized for several years as the major pest of wheat, that attack annually the most wheat-growing regions in Morocco. The damage caused by this insect can go up to the total destruction of culture, especially if the infestation coincides with the early stage of the plant (Lhaloui *et al.,* 2005). To overcome this problem several methods are used but the genetic control, through the introduction of the resistance in varieties, is the most effective and economical approach for control the damage caused by this insect (Lhaloui *et al.,* 2005; Nasrellah and Lhaloui 2006). So far, 34 major HF resistance genes have been identified, named and characterized (Liu *et al.,* 2005; McIntosh *et al.,* 2005; Chunlian *et al.*, 2013).

Leaf rust caused by *Puccinia triticina*, stripe rust caused by *Puccinia striiformis* and stem rust caused by *Puccinia graminis* are the major foliar diseases of wheat, resulting in yield loss all over the world (Kaur *et al.*, 2008). The wheat cultivars become susceptible to rusts due to their narrow genetic base for resistance and the rapid rate evolution of the pathogen, making it necessary to search for new sources of resistance. So far, nearly 58 leaf rust and 40 stripe rust resistance genes have been identified and designated as *Lr1* through *Lr58* and *Yr1* through *Yr40*, respectively (McIntosh *et al.,* 2005; Kuraparthy *et al.,* 2007).

Drought is one of the most important abiotic stress factor limiting crop yields around the world. The increase in global temperature, drought stress or water shortage is projected to have a growing impact on plants and crop production (Kiliç and Yağbasanlar, 2010). The ability of a cultivar to produce high and satisfactory yield over a wide range of stress and non-stress environments is very important (Ahmad *et al.,* 2003). The response of plants to water stress depends on several factors such as developmental stage, severity of stress and cultivar genetic (Beltrano and Marta, 2008).

In this study, we present a practical validation of the breeding strategy to produce wheat lines derived from elite cultivars with several characteristics. Molecular markers were used to screen double hybrid (DHy) lines produced from a cross between three wheat varieties considered as donor parents: *Dharwar*, *Annuello* and *Stylet* crossed with six varieties considered as recurrent parents: *Achtar*, *Aguilal*, *Merchouch*, *Baraka*, *Salama* and *Amal*. Following the phenotypic selection (PS) was applied for the doubled haploid (DH) plants to select new genotypes with rust resistance genes, HF resistance genes, drought tolerance gene and grain protein content.

Materials and methods

Plant materials

All bread wheat (*Triticum aestivum* L. var. *aestivum*, 2n = 6x = 42, genome *AABBDD*) cultivars analyzed in this work was obtained from the Laboratory of Plant Biotechnology at Regional Center for Agricultural Research (CRRA), INRA, Settat, Morocco. To achieve our crosses, we selected three wheat varieties as donor parents: *Dharwar*, *Annuello* and *Stylet* crossed with six varieties considered as recurrent parents: *Achtar*, *Aguilal*, *Merchouch*, *Baraka*, *Salama* and *Amal* (Table 1). The exotic cultivars are used in this experiment for transfer of important agronomically genes to the Moroccan varieties to improve their tolerance to biotic and abiotic stresses.

Table 1. Origin, pedigree and genetic characteristics of nine bread wheat cultivars used in this experiment as donor or recurrent parents.

Cultivar name	Origin	Pedigree	Genetic characteristics	Reference
Stylet	Australian variety	Molineux/2*Trident	Rust resistance gene (*Lr37/Sr38/Yr17*)	Kuchel *et al.* (2007); McIntosh *et al.*
Annuello	Australian variety	Pavon(SIB)/TM-56 (VF-665)//Janz	Rust resistance gene (Lr34/Yr18 ; *Lr24/Sr24*) ; glutenin allele (*Glu-A3*)	Kuchel *et al.* (2007); McIntosh *et al.* (2011)
Dharwar	Indian variety	Unknown	Drought tolerance gene	Noorka and Schwarzacher (2013)
Aguilal	Moroccan variety	Saïs*2/1/KS-85-14-2	HF resistance gene (*H22*); height reducing genes	Wheat Atlas (2014)
Achtar	Moroccan variety	Hork/1/Yamhill/2/Kalyansona/1/Bluebird	Height reducing genes	Wheat Atlas (2014)
Amal	Moroccan variety	Bobwhite/1/Buckbuck	Height reducing genes	Wheat Atlas (2014)
Baraka	Moroccan variety	Vicam-71/2/Ciano-671/Siete-Cerros-66/3/ Kalyansona/1/Bluebird	Height reducing genes	Wheat Atlas (2014)
Merchouch	Moroccan variety	Kalyansona/1/Ciano/2/815 6²/3/BT908	Height reducing genes	Wheat Atlas (2014)
Salama	Florimond Desprez	Introduced from France by SONACOS, Morocco	No information	ONSSA, Morocco (2015)

Breeding scenario

Breeding strategy was started in 2011 to improve rust resistance, HF resistance, drought tolerance and end-use quality. *Stylet* cultivar was used for the introgression of rust resistance genes *Lr37/Sr38/Yr17* and *Annuello* cultivar was chosen as the donor of rust resistance genes *Lr34/Yr18* and *Lr24/Sr24*, also the donor of a glutenin allele *Glu-A3* for improved end-use quality. *Dharwar* cultivar was chosen for the drought tolerance gene.

Simple hybrids were developed from cross between three cultivars of bread wheat considered as donor parents: *Dharwar*, *Annuello* and *Stylet* with six recurrent parents: *Achtar*, *Aguilal*, *Merchouch*, *Baraka*, *Salama* and *Amal*. The following crosses were made by manual emasculation and pollination in the greenhouse to get the wheat hybrids. Then another cross was made between different simple hybrids to produce double hybrid lines.

The technology of anther culture is used in many cereal breeding programs, and is more cost-effective than intergeneric crosses in the production of doubled haploid (DH) plants. It's necessary to provide resistance genes and to produce homozygous lines. Anthers of the hybrid plants were cultivated on C17 medium (Wang and Chen, 1986), and 100 plants were regenerated on MS medium (Murashige and Skoog, 1962). Albinos and abnormal plants were eliminated, and haploid green plants were diploidized by treating them with colchicine solution (0.2%). A total of 40 DH lines were developed. The seeds of these genotypes were first multiplied in the greenhouse of CRRA-Settat and then in the field of INRA Research Experimental Station at Sidi Al Aydi, during the 2014-

2015 growing seasons. The details of this breeding strategy are shown schematically in the Fig. 1.

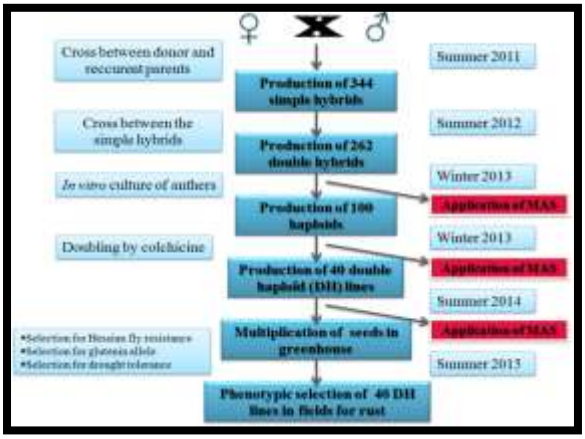


Fig. 1. Diagrammatic representation of the breeding scenario used in this experiment from the 2011 to 2015 year.

Phenotypic characterization

Evaluation of Hessian fly resistance

This evaluation was conducted in greenhouse of Entomology Laboratory of CRRA-Settat, Morocco. Two weeks before the artificial infestation, the stored plants containing the adults HF are removed from the refrigerator for up the diapause. The DH lines were seeded in greenhouse and maintained at natural daylight settings (20 ± 2°C). Each flat contained one HF resistant cultivar, *Saada*, and one susceptible cultivar, *Nasma*, as checks. When plants were at the two-leaf stage, they are infested by about 100 newly mated HF females in each flat within a cheesecloth tent. Three weeks after infestation, the seedlings were examined to identify susceptible and resistant phenotypes. Susceptible plants were stunted with dark green leaves and live larvae. Resistant plants grew normally with light green leaves and dead larvae (Fig. 2). The reaction was confirmed under the microscope for the presence of live or dead larvae.

Evaluation of rust resistance

This evaluation was conducted at INRA Research Experimental Station in Sidi Al Aydi, Morocco (a low-rainfall rainfed wheat production zone with an annual average rainfall of 300 mm; altitude 230 m, lat. 33.17° N, long. 7.40° W). The soil is a vertic calcixeroll and has a depth of 90 to 120 cm. Available in horizontal lines a collection of 40 DH genotypes tested in the field for rust resistance. The number of lines is 40 and each line contains 200 seeds from each DH genotypes. Weeds were removed manually. It presents the results of host-pathogen interactions and indicates whether the host manifests a reaction of resistance or sensitivity, the degree of attack of the disease.

Evaluation of end-use quality

Glutenins were extracted from wheat grains according to Singh *et al.* (1991) with minor modifications. A single wheat grain is crushed into fine powder and 20 mg of flour was used for extraction. The water soluble proteins and monomeric gliadins were first removed by three time extractions with 50% 2-propanol for 30 min at 65°C. Subsequently, glutenins were extracted from the residues with 100 µl extraction buffer, including 50% 2-propanol, 80 mM Tris-HCl pH 8.0 with freshly added 1% (w/v) dithiothreitol (DTT) by stirring at 65°C for 30 min, and then alkylated by stirring with an equal volume of extraction buffer replacing 1% DTT with freshly added 1.4% (v/v) 4-vinylpyridine under the same water incubation conditions. After centrifugation at 10 000 g for 10 min, 100 µl of the supernatant was moved to new tubes for SDS-PAGE analysis. Sample buffer containing 2% SDS, 0.02% bromophenol blue, 0.08 M Tris-HCl pH 8.0 and 40% glycerin was mixed with the same volume of protein sample. The mixture was incubated at 65°C for 15 min. After centrifugation at 10 000 g for 2 min, protein samples were electrophoresed at 20mA, and the gel was incubated 1h with methanol-acetic acide-water (4 :1 :5) and then stained overnight with 15% (w/v) trichloroacetic acid and 1% (w/v) Coomassie Brilliant Blue R250. The gel was destained with 10% acetic acid. The alleles for high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) were named according to Payne and Lawrence (1983) and Nieto-Taladriz *et al.* (1997), respectively. The *Chinese Spring* cultivar with known HMW-GS composition was included as standards (Yan *et al.,* 2003; Lan *et al.,* 2013).

Evaluation of drought tolerance

The grains were germinated in pots placed in greenhouse, they are regularly irrigated until the fourth-leaf-stage, and the water stress treatment is applied by stopping irrigation until the different water stress levels (T0=100%, T1=60% and T2=30% of retention capacity). Weighing pots and adjust their moisture content was performed every three days and no fertilizer was applied at the end to see better the behavior of the genotypes in response to water stress.

- *Number of leaves, leaf length (cm)* and *root length (cm)* were determined.
- *Leaf area (cm²)* to the third leaf is determined by the method of Paul *et al.* (1979).
- *The relative water content (%)* is one of the evaluation criteria of tolerance to drought, proposed by Clark and Macgaig (1982). It's calculated by the formula of Barrs (1968).
- *The content of chlorophyll* is determined by the method of Rao and Le Blanc (1965).
- *Total soluble carbohydrates (g/100mg)* are assayed by the method of Dubois *et al.* (1956).
- *Statistical Analysis:* SPSS software was used to analyze obtained data. Analysis of variance, simple chi-square (χ2) test and analysis of correlation were performed.

Results and discussion

Validation of selection for Hessian fly resistance

The tested DH lines showed variable reactions to infestation in the field as well as in the greenhouse (Fig. 2). The result of the resistance to HF is shown in table 2. Used the MAS selection, 2.56% carries the resistance gene but for the phenotypic selection in the field 7.69% are resistant to infestation by the HF and 10.25% for the phenotypic selection in the greenhouse.

Only one line, *11DHBW31* produced from the cross '*Stylet/Baraka/2/ Annuello/Aguilal*', is 100% resistant to HF by the MAS selection and the phenotypic selection in greenhouse and in the field. Similar tests conducted, previously, in Tunisia by Bouktila *et al.,* 2005, indicated a high level of resistance conferred by three genes of HF (*H5*, *H11* and *H13)* in both field and greenhouse conditions.

Fig. 2. The symptoms of artificial infestation by Hessian fly observed during the phenotypic selection made in greenhouse of the Laboratory of Entomology (CRRA-Settat).

The difference between the MAS selection and the phenotypic selection was estimated by ANOVA test using LSD ($P<0.05$). The ANOVA test showed significant differences for the percentage of susceptible plants between each genotype carrying a resistance gene and the susceptible check *Nasma* carrying no resistance gene.

In order to investigate the extent of relatedness between results obtained by MAS and by phenotypic selection in field and in greenhouse, a Spearman rank correlation analysis was performed, which equaled 0.333.

This implied that results of both tests were positively correlated. Results indicated that these varieties should be included in breeding programs aiming to transfer Hessian fly resistance genes into high yield varieties used by farmers in Morocco.

Validation of selection for rust resistance

Data presented in table 3 illustrates rust resistance genes identified in the DH lines using molecular markers to detect the presence of resistance genes for leaf rust, yellow rust and stem rust. Altogether 40 DH lines of the nine parent combinations were analyzed and selected for the rust resistance in the

Experimental Station at Sidi Al Aydi during the 2014/2015 agricultural campaign.

Table 2. Presence of resistance genes to Hessian fly in the doubled haploid lines of bread wheat used in this experiment (***, **, * significant effect 0.001, 0.01, 0.05).

DH lines	Pedigree	MAS	PS in greenhouse	PS in field
11 DHBW 31	*Stylet/Baraka/2/Annuello/Aguilal*	+	R	R
11 DHBW 26	*Stylet/Aguilal/2/Annuello/Aguilal*	-	R	R
11 DHBW 21	*Stylet/Annuello/2/Annuello/Aguilal*	-	R	S
11 DHBW 20	*Stylet/Annuello/2/Annuello/Aguilal*	-	R	R
	ANOVA test		0.324*	
Spearman Rank	Correlation		0.333*	

(+) presence of gene; (–) absence of gene; (R) resistance; (S) susceptible; (PS) phenotypic selection; (MAS) marker assisted selection.

Our present study revealed that among 40 entries tested, only 19 showed resistances of which 6 were resistant to both leaf rust and yellow rust; yellow rust and stem rust or leaf rust and stem rust. Used the MAS selection, 2.56% carries the resistance gene for leaf rust, 38.46% carries the resistance gene for yellow rust, and stem rust was present in just 15.38% of genotypes analyzed. These types of studies to validate the presence of rust resistance genes with molecular markers have also been conducted by other workers (Datta *et al.*, 2011; Pal *et al.*, 2015). For the phenotypic selection, we noticed almost the same results observed for genotypic selection. 2.56% are resistant to leaf rust, 38.46% are resistant to yellow rust and for stem rust the result are not observed in the field.

The difference between the genotypic and phenotypic selection was estimated by ANOVA test using LSD ($P<0.05$). The ANOVA test showed significant differences between each genotype carrying a resistance gene and the susceptible carrying no resistance gene.

In order to investigate the extent of relatedness between results obtained by MAS and by phenotypic selection in field and in greenhouse, a Spearman rank correlation analysis was performed, which equaled 0.016 for leaf rust and 0.065 for yellow rust. This implied that results of both tests were positively correlated. Results indicated that these varieties should be included in breeding programs aiming to transfer rust resistance genes into high yield varieties used by farmers in Morocco.

Table 3. Presence of resistance genes to leaf, yellow and stem rust in the doubled haploid lines of bread wheat used in this experiment (***, **, * significant effect 0.001, 0.01, 0.05).

DH lines	Pedigree	MAS	PS for Leaf Rust	MAS	PS for Yellow Rust	MAS	PS for Stem Rust
11 DHBW 1	*Dharwar/Aguilal/2/Annuello/Aguilal*	-	S	+	R	-	Results not observed at a field
11 DHBW 5	*Stylet/Annuello/2/Annuello/Salama*	-	S	+	R	-	
11 DHBW 10	*Stylet/Baraka/2/Annuello/Aguilal*	+	R	-	S	+	
11 DHBW 11	*Stylet/Annuello/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 13	*Dharwar/Aguilal/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 15	*Dharwar/Aguilal/2/Annuello/Baraka*	-	R	-	S	-	

DH lines	Pedigree	MAS	PS for Leaf Rust	MAS	PS for Yellow Rust	MAS	PS for Stem Rust
11 DHBW 16	*Dharwar/Aguilal/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 17	*Stylet/Merchouch/2/Annuello/Merchouch*	-	R	-	S	-	
11 DHBW 19	*Stylet/Baraka/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 20	*Stylet/Annuello/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 21	*Stylet/Annuello/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 23	*Stylet/Annuello/2/Annuello/Salama*	-	S	+	R	-	
11 DHBW 24	*Stylet/Aguilal/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 25	*Stylet/Baraka/2/Annuello/Aguilal*	-	S	+	R	+	
11 DHBW 26	*Stylet/Aguilal/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 27	*Stylet/Baraka/2/Annuello/Aguilal*	-	S	+	R	+	
11 DHBW 29	*Stylet/Annuello/2/Annuello/Salama*	-	S	-	S	+	
11 DHBW 31	*Stylet/Baraka/2/Annuello/Aguilal*	-	S	+	R	-	
11 DHBW 32	*Stylet/Baraka/2/Annuello/Aguilal*	-	S	+	R	-	
	ANOVA test		0.324*		0.001***		----
Spearman rank			0.016**		0.218*		----

(+) presence of gene; (–) absence of gene; (R) resistance; (S) susceptible; (PS) phenotypic selection; (MAS) marker assisted selection.

Validation of selection for end-use quality

Wheat end-use quality is an important trait in breeding and is evaluated by different physical, biochemical and rheological assays. Major quality traits are discussed including grain and flour protein and ash concentration, dough strength and extensibility, starch composition, grain hardness, and end-use product color. These traits are controlled by different genes, such as *Glu* and *Gli* loci (Zhen *et al.,* 2014).

The polymeric glutenins are further subdivided into high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) (Cornish *et al.*, 2001; Zhen *et al.*, 2014). LMW-GS strongly influence the bread-making quality of bread wheat and play a major role in determining dough resistance and extensibility. Among different alleles of all loci for LMW glutenin subunits, *Glu-A3* has the best extensibility in wheat (Zhang *et al.*, 2012). The electrophoretic separation of LMW-GS and HMW-GS components of the 9 cultivars of bread wheat is presented in Fig. 3 and the *Chines Spring* cultivar is used as control.

The *Salama* and *Amal* cultivars has the same HMW-GS '2*; 7+9; 5+10'. The *Merchouch* and *Achtar* cultivars has also the same HMW-GS '2*; 17+18; 5+10'. The *Dharwar* and *Chines Spring* cultivars has also the same HMW-GS 'Null; 7+8; 2+12'. *Stylet* cultivar has the HMW-GS '1; 7+9; 5+10', *Annuello* cultivar has '1; 7+8; 2+12' and *Baraka* cultivar has 'Null; 17+18; 2+12'.

SDS-PAGE is one method for identification of allelic components in quality scoring of wheat cultivars, but in this system the mobility of subunits does not exactly correspond with the size and sometimes makes interpretation of banding pattern difficult. However, MAS can help avoid misinterpretation of results from SDS-PAGE. Then the PCR-based DNA markers can be used for distinguishing and screening of good or poor bread-making quality in wheat.

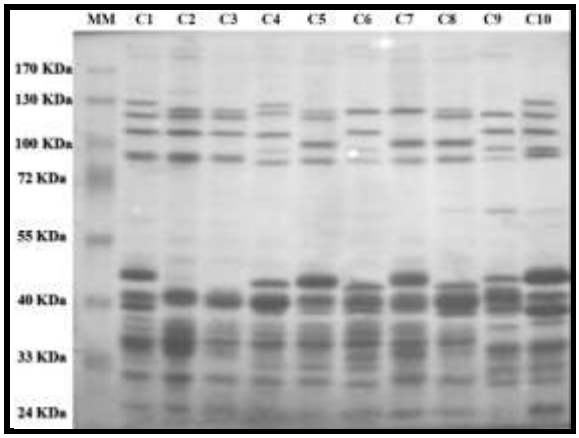


Fig. 3. SDS-PAGE separation of the glutenins component in the 10 cultivars used.
C1 (*Stylet*); C2 (*Salama*); C3 (*Amal*); C4 (*Annuello*); C5 (*Merchouch*); C6 (*Chines Spring*); C7 (*Baraka*); C8 (*Achtar*); C9 (*Dharwar*); C10 (*Aguilal*).

The allelic composition for each DH lines subjected to this study is summarized in table 4.

For the HMW-GS, the results of this work showed that 25% of the bread wheat genotypes studied possess the 'null' subunit at *Glu-A1* loci, 60% contained HMW-GS '1' and 15% of the genotypes which possesses the HMW-GS '2*'. At *Glu-B1*, results showed that the majority (60%) of genotypes possesses subunits '7+8'; the three DH lines *11DHBW38*, *11DHBW6*, *11DHBW52* possess subunits '7+9'; the two genotypes *11DHBW10*, *11DHBW18* possesses subunit '17+18' and the genotypes *11DHBW23*, *11DHBW40* possess subunits '7'.

For the *Glu-D1* loci, results showed that the majority (65%) of genotypes possesses subunits '2+12' and the minority (35%) possesses subunits '5+10'. Concerning LMW-GS coded at *Glu-A3* loci, 55% of genotypes posses LMW2 related with good wheat gluten elasticity, whereas LMW1 related with poor wheat gluten elasticity (Payne *et al.,* 1984), are present only in 45% of genotypes.

More recently, studies on the effects of different prolamins alleles on wheat quality properties revealed positive effects of the HMW-GS subunit '1' on gluten quality (Martinez *et al.*, 2005).

In our results the composition of HMW-GS showed that the most frequent allele in *Glu-A1* is the '1' allele followed by 'null' and '2*', and in *Glu-B1*, the most frequent band is '7+8'. The prevalence of LMW2 in the genotypes studied may reflect the success of our study on the creation of varieties with very good technological quality.

Table 4. Presence of glutenin genes and their composition in the doubled haploid lines of bread wheat used in this experiment (***, **, * significant effect 0.001, 0.01, 0.05).

DH lines	Pedigree	MAS	High and low molecular weight subunits of glutenin
11 DHBW 1	*Dharwar/Aguilal/2/Annuello/Aguilal*	+	GS-HMW : Null ; 7 + 8 ; 5 +10 (c, b, d) GS-LMW: LMW 2
11 DHBW2	*Dharwar/Aguilal/2/Annuello/Aguilal)*	-	GS-HMW :1 ; 7 +8 ; 2 +12 (a, b, a) GS-LMW: LMW 2
11 DHBW 3	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : Null ; 7 +8; 2 +12 (c, b, a) GS-LMW: LMW 2
11 DHBW 5	*Stylet/Annuello/2/Annuello/Salama*	+	GS-HMW : 1; 7 +9; 2 +12 (a, c, a) GS-LMW: LMW2
11 DHBW 6	*Stylet/Annuello/2/Annuello/Salama*	+	GS-HMW : 1; 7 + 9; 2 +12 (a, b, a) GS-LMW: LMW 2
11 DHBW 7	*Stylet/Annuello/2/Annuello/Salama*	+	GS-HMW : 1; 7 + 8; 5 + 10 (a, b, d) GS-LMW: LMW2

DH lines	Pedigree	MAS	High and low molecular weight subunits of glutenin
11 DHBW 10	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : 1; 17 + 18; 2 +12 (a, i, a) GS-LMW: LMW 2
11 DHBW 13	*Dharwar/Aguilal/2/Annuello/Aguilal*	+	GS-HMW : 2*; 7 +8; 5 + 10 (b, b, d) GS-LMW: LMW2
11 DHBW 17	*Stylet/Merchouch/2/Annuello/Merchouch*	+	GS-HMW : 1; 7 +8; 5 + 10 (a, b, d) GS-LMW: LMW 2
11 DHBW 18	*Stylet/Merchouch/2/Dharwar/Aguilal*	+	GS-HMW : 2*; 17 + 18; 2 +12 (b, i, a) GS-LMW: LMW2
11 DHBW 19	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : Null; 17 + 18; 2 +12 (c, i, a) GS-LMW: LMW2
11 DHBW 23	*Stylet/Annuello/2/Annuello/Salama*	+	GS-HMW : 1; 7; 5 + 10 (a, a, d) GS-LMW: LMW 2
11 DHBW 25	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : 1; 7 +8; 2 +12 (a, b, a) GS-LMW: LMW 2
11 DHBW 27	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : 1; 7 +8; 2 +12 (a, b, a) GS-LMW: LMW 2
11 DHBW 32	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : 2*; 7 +8; 5 +10 (b, b, d) GS-LMW: LMW 2
11 DHBW 35	*Dharwar/Aguilal/2/Annuello/Aguilal*	+	GS-HMW : 1; 7 +8; 5 + 10 (a, b, d) GS-LMW: LMW 2
11 DHBW 37	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : Null; 7 +8; 2 +12 (c, b, a) GS-LMW: LMW 2
11 DHBW 38	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : Null; 7 + 9; 2 + 12 (c, c, a) GS-LMW: LMW 2
11 DHBW 39	*Stylet/Baraka/2/Annuello/Aguilal*	+	GS-HMW : 1; 7 +8; 2 +12 (a, b, a) GS-LMW: LMW 2
11 DHBW 40	*Stylet/Merchouch/2/Dharwar/Aguilal*	+	GS-HMW : 1; 7 ; 2 +12 (a, a, a) GS-LMW: LMW 2
	ANOVA test		0.001***
Spearman rank			- 0.093**

(+) presence of gene; (–) absence of gene; (HMW) high molecular weight; (LMW) low molecular weight; (MAS) marker assisted selection.

Validation of selection for drought tolerance

Drought is one of the prevalent environmental conditions that cause adverse effects on the growth of plants. It's the most severe stress, limits plant growth and field crops production more than any other environmental stresses (Kamran *et al.*, 2014). In this experiment, we have used *Dharwar* as an extremely drought tolerant spring wheat variety. This cultivar is being crossing with other cultivar of bread wheat to achieve for the selection of new genotypes tolerant to drought.

In the table 5 we presented all genotypes arising from the crossing with the *Dharwar* parent. The results of

this study show that drought affects negatively leaf length, root length and leaf area (Fig.4). They also show varietal differences in response between the different genotypes and interaction genotype x drought. They corroborate the result of other studies including those of Ahmad *et al.* (2003) and of Kiliç and Yağbasanlar (2010).

Fig. 4. Effect of water stress condition on the development of doubled haploid lines of bread wheat made in the greenhouse of Laboratory of Plant Biotechnology (CRRA-Settat).

Drought stress has a significant effect on the content of chlorophyll a and b, and on the total of chlorophyll ($p<0.01$). It has a significant effect on the rate chlorophyll a+b ($p<0.05$). Decrease of chlorophyll a+b content, was lowest in *11DHBW35* (produced from the cross '*Dharwar/Aguilal/2/Annuello/ Aguilal*') with 23.52 ± 0.47 value for the To and 15.27 ± 0.61 for the T2, the highest value observed in *11DHBW40* (produced from the cross *'Stylet/Merchouch/2/Dharwar/Aguilal'*) with 43.39 ± 0.29 for To and 27.17 ± 0.17 for T2.

As the same of chlorophyll a, content of chlorophyll b decreased under effect of drought stress. Chlorophyll content was an indicator of drought tolerance and it could be used as screening tool for drought tolerance in wheat.

According to Mohammadi *et al.* (2009) water stress condition caused reduction in chlorophyll content. Tolerant genotypes of wheat had higher chlorophyll content than sensitive genotypes (not crossed with Dharwar cultivar) under the drought. The wheat genotypes with high chlorophyll content can produce high yield under moisture-stressed conditions and there was a significant positive correlation between chlorophyll content and yield (Kamran *et al.*, 2014).

In this research increase of drought stress caused a significant ($p<0.05$) increase in total soluble carbohydrate content. The mean comparison of carbohydrate content in the different DH lines represented shows that *11DHBW2* (produced from the cross *'Dharwar/Aguilal/2/Annuello/Aguilal'*) had the most amount and *11DHBW16* (produced from the cross *'Dharwar/Aguilal/2/Annuello/Aguilal'*) had the lowest amount. In various DH lines, the mean comparison of RWC showed that *11DHBW35* (produced from the cross *'Dharwar/Aguilal/2/ Annuello/Aguilal'*) with 76.43 ± 2.58% value had highest RWC and *11DHBW2* (produced from the cross '*Dharwar/Aguilal/2/Annuello/Aguilal*') had lowest value (47.44 ± 1.40%).

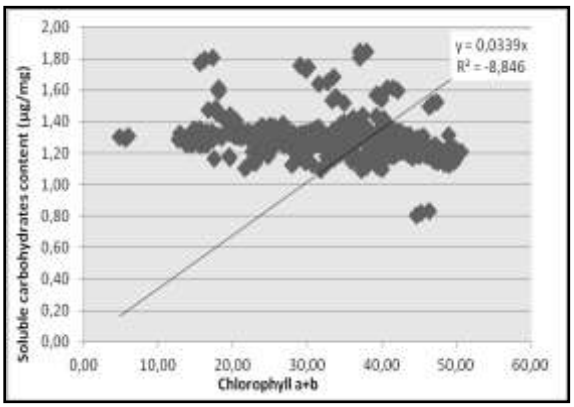
Table 5. Effects of water stress conditions on physiological and morphological parameters of doubled haploid lines of bread wheat studied (***, **, * significant effect 0.001, 0.01, 0.05).

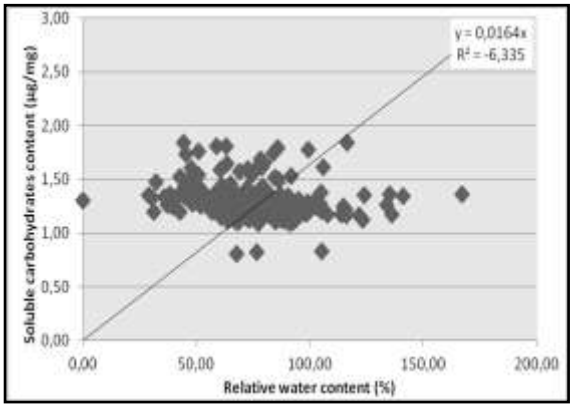
DH lines	Pedigree	Leaf length	Root length	Leaf area	RWC	Chlorophyll content	Carbohydrate content
11 DHBW 1 (To)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	30.00 ± 0.57	32.67 ± 0.82	18.37 ± 0.72	78.75 ± 3.50	22.23 ± 0.41	1.11 ± 0.08
11 DHBW 1 (T2)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	30.00 ± 0.57	19.33 ± 0.88	16.12 ± 0.66	48.41 ± 2.54	17.44 ± 0.32	1.47 ± 0.12

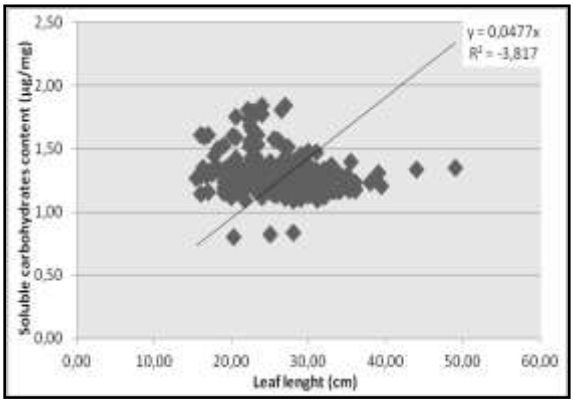
DH lines	Pedigree	Leaf length	Root length	Leaf area	RWC	Chlorophyll content	Carbohydrate content
11 DHBW 2 (To)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	19.43 ± 0.29	15.00 ± 0.57	19.04 ± 0.03	63.42 ± 1.96	44.02 ± 0.55	1.19 ± 0.12
11 DHBW 2 (T2)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	22.70 ± 0.35	21.00 ± 1.52	15.53 ± 0.06	47.44 ± 1.40	34.01 ± 0.46	1.53 ± 0.03
11 DHBW 13 (To)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	27.00 ± 1.02	26.37 ± 0.32	18.29 ± 0.02	90.80 ± 3.04	44.62 ± 0.29	1.21 ± 0.11
11 DHBW 13 (T2)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	26.33 ± 0.46	34.30 ± 0.27	14.45 ± 0.26	49.97 ± 3.88	31.47 ± 0.27	1.34 ± 0.01
11 DHBW 15 (To)	*Dharwar/Aguilal/2 /Annuello/Baraka*	21.93 ± 0.96	18.83 ± 1.09	20.45 ± 0.32	73.72 ± 3.52	41.85 ± 0.16	1.19 ± 0.16
11 DHBW 15 (T2)	*Dharwar/Aguilal/2 /Annuello/Baraka*	23.00 ± 0.28	35.67 ± 1.20	17.04 ± 0.03	54.75 ±2.09	34.65 ± 0.15	1.38 ± 0.03
11 DHBW 16 (To)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	24.53 ± 0.42	18.83 ± 0.32	19.37 ± 0.07	78.97 ± 1.18	45.27 ± 0.19	1.20 ± 0.18
11 DHBW 16 (T2)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	24.37 ± 0.36	31.13 ± 0.96	16.09 ± 0.09	60.06 ± 2.78	35.61 ± 0.27	1.31 ± 0.02
11 DHBW 35 (To)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	31.80 ± 0.41	22.47 ± 0.24	23.89 ± 0.05	81.56 ± 2.02	23.52 ± 0.47	1.21 ± 0.13
11 DHBW 35 (T2)	*Dharwar/Aguilal/2 /Annuello/Aguilal*	27.07 ± 0.06	37.00 ± 0.11	18.53 ± 0.12	76.43 ± 2.58	15.27 ± 0.61	1.33 ± 0.53
11 DHBW 40 (To)	*Stylet/Merchouch/2 /Dharwar/Aguilal*	28.43 ± 0.22	12.60 ± 0.31	22.42 ± 0.05	78.84 ±2 .26	43.39 ± 0.29	1.22 ± 0.01
11 DHBW 40 (T2)	*Stylet/Merchouch/2 /Dharwar/Aguilal*	24.23 ± 0.23	32.07 ± 0.63	19.45 ± 0.26	62.18 ± 2.01	27.17 ± 0.17	1.34 ± 0.04
Dharwar To		35.97 ± 1.99	20.00 ± 0.00	23.09 ± 0.06	71.41 ± 1.43	49.47 ± 0.23	1.21 ± 0.01
Dharwar T2		29.00 ± 1.02	17.50 ± 0.50	16.59 ± 0.04	63.73 ± 1.68	34.51 ± 0.18	1.34 ± 0.03
Simple chi-square ($\chi 2$) test		0.106*	0.381*	0.329*	0.337*	0.307*	0.118*
ANOVA Test		0.585	0.087**	0.011**	0.032**	0.082**	0.002***

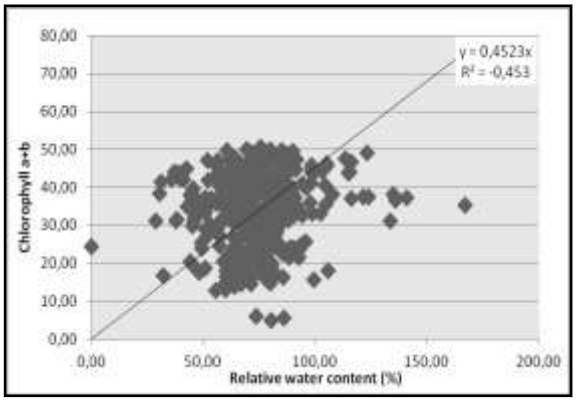
In fact, RWC, in drought stress decreased in all under testing DH lines and the same results of this test have reported in beans (Keyvan, 2010). On the other hand, difference in RWC of different genotypes that are under drought stress may be for this reason that the ability of more absorption of water from soil or ability of stomata to reduce the loss of water is different. RWC was the best criteria for classification and screening of drought tolerant genotypes (Hasheminasab *et al.,* 2012).

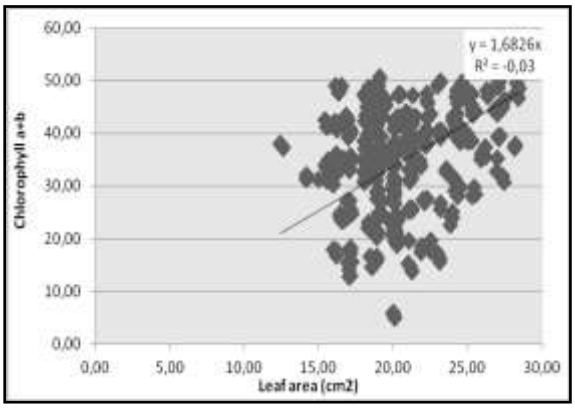
The study of the Pearson correlation between physiological and morphological parameters studied in 40 DH lines of bread wheat studied under water stress conditions is presented in table 6 and Fig. 5. This study allowed us to infer the existence of a significant positive or negative linear correlation between number of leaves, leaf length, root length, leaf area, relative water content, chlorophyll a+b content and carbohydrate content.

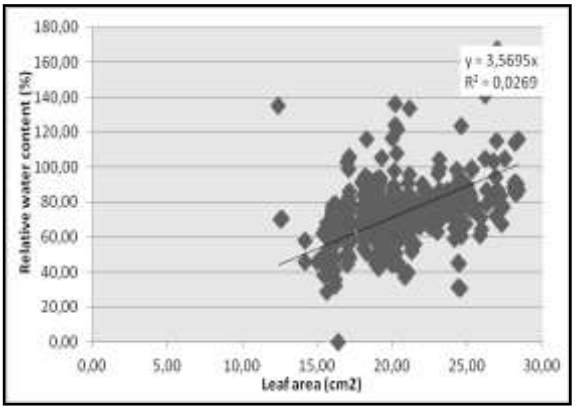












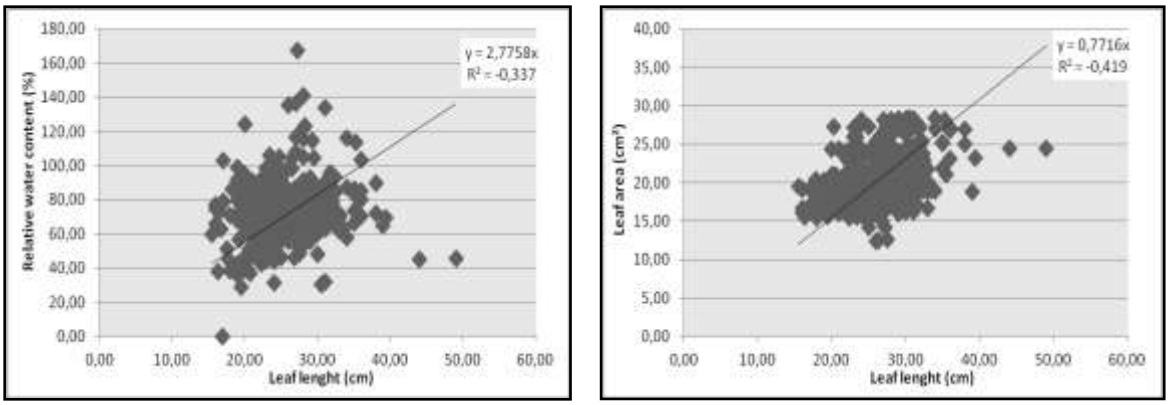


Fig. 5. Positive correlations between physiological and morphological parameters of the 40 doubled haploid lines of bread wheat studied under water stress conditions.

Table 6. Pearson correlation determined from physiological and morphological parameters of 40 doubled haploid lines of bread wheat studied under water stress conditions.

	Number of leaves	Leaf length	Root length	Leaf area	RWC	Chlorophyll content	Carbohydrate content
Number of leaves	1						
Leaf length	0.016	1					
Root length	-0.104*	-0.046	1				
Leaf area	0.218**	0.353**	-0.040	1			
RWC	0.079	0.145**	-0.050	0.359**	1		
Chlorophyll content	0.067	0.019	-0.185**	0.263**	0.143**	1	
Carbohydrate content	-0.270**	-0.197**	0.182**	-0.353**	-0.206**	-0.288**	1

* The correlation is significant at 0.05; ** The correlation is significant at 0.01.

Gupta *et al.* (2001) reported positive correlations among plant height, leaf area and gain yield at boot and anthesis stages in wheat cultivars. Some studies show that leaf chlorophyll content is positively correlated with photosynthetic capacity. It is reasonable to assume that high chlorophyll capacity of wheat plants under drought conditions could be identified by selecting breeding materials with high chlorophyll capacity. Chlorophyll content was useful trait for selecting drought tolerant wheat genotypes (Farshadfar *et al.*, 2012).

It is concluded from the results of this study that water stress reduced some morphological and physiological components in doubled haploid lines of bread wheat. The differential response of genotypes imposed water stress condition indicates that the wheat genotypes originating from crossing with *Dharwar* cultivar are more tolerant to drought than others. On an overall, our results and the findings of others (Gupta *et al.*, 2001; Keyvan, 2010; Farshadfar *et al.*, 2012; Hasheminasab *et al.,* 2012) show that a strategy of selecting should take into consideration others growing period of plants (early flowering, long grain filling period and late maturity period).

Conclusion

The aim of this study was to improve the disease resistance, the drought tolerance and grain quality of an elite recurrent parent through marker assisted gene introgression. We have shown in a pragmatic breeding strategy that selection with molecular markers has resulted in the production of a number of doubled haploid lines with improved rust resistance, HF resistance, drought tolerance and end-use quality.

Results presented here led to the conclusion that marker-assisted selection offers the opportunity to select desirable lines on the basis of genotype rather than phenotype, especially in the case of combining different genes in a single genotype. With the help of molecular marker, the pyramiding of disease resistance genes should facilitate more efficient breeding and the phenotypic selection is necessary to finish the breeding strategy.

Abbreviation

CRRA: Regional Center for Agricultural Research, DH: Doubled Haploid, DHy: Double Hybrid, HMW: High Molecular Weight, GS glutenin subunits, HF: Hessian fly, LMW: Low Molecular Weight, MAS: Marker Assisted Selection, PS: Phenotypic Selection, RWC: Relative Water Content.

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